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Multidrug-resistant *Myobacterium tuberculosis* caused by the Beijing genotype and a specific T1 genotype clone (SIT No. 266) is widely transmitted in Minsk

Aksana Zalutskaya [a], *Maria Wijkander* [b], *Pontus Jureen* [b], *Alena Skrahina* [a], *Sven Hoffner* [b,*]

[a] *Republican Scientific and Practical Centre for Pulmonology and Tuberculosis, Minsk, Belarus*
[b] *Swedish Institute for Communicable Disease Control, Solna, Sweden*

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ABSTRACT

Setting: This study was performed in the city of Minsk in Belarus, where a very severe problem with MDR-TB was demonstrated in a recent drug resistant survey.
*Objective*The aim of this study was to use molecular typing of MDR and pan-susceptible clinical isolates of *Mycobacterium tuberculosis* to increase the understanding of the transmission patterns and possible differences between the strains causing susceptible and drug-resistant tuberculosis.
Study population and methods: Consecutive isolates from pulmonary TB patients in Minsk were collected at the Belarusian National Reference Laboratory. Isolates found to be either pan-susceptible or MDR were included in the study, which totally comprised 81 MDR and 82 pan-susceptible clinical isolates. All isolates were characterized by spoligotyping. The major clusters were characterized using sequencing of the *pncA* gene.
Results: Three out of four MDR cases were caused by one out of two drug-resistant clones of *M. tuberculosis* belonging to the Beijing and T1 genotypes, respectively. A single T1 clone, SIT No. 266, found exclusively in the MDR cohort, was shown to cause no less than 30% of all MDR-TB cases.
Discussion: The findings indicate that the major cause of MDR-TB in Minsk is an ongoing transmission of certain already resistant M. tuberculosis strains.
Conclusion: The significant transmission of MDR-TB in Minsk underlines the urgent need for strengthened infection control measures to limit the transmission in order to better control MDR-TB.

Introduction

Multidrug resistant tuberculosis (MDR-TB) is a severe threat to effective TB-control as well as to successful treatment of the individual patients. It is estimated by the WHO that globally more than 500,000 TB patients are infected with strains resistant to the most effective anti-TB drugs–rifampicin (RIF) and isoniazid (INH) – and thus classified as MDR-TB cases. Most of these are found in India or China, but it is clearly documented [1] that the highest incidence with up to around 20% of new and 60% of re-treatment cases is to be found in Eastern Europe and especially in some of the countries of the former Soviet Union (USSR).

Belarus, a country in the western part of the former USSR and neighbor of the EU countries Poland, Latvia and Lithuania, is no exception. Even though the reported TB incidence (45/

* Corresponding author. Tel.: +46 84572431.
E-mail address: sven.hoffner@smi.se (S. Hoffner).
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100,000) is lower than in its neighboring countries, the high and increasing level of MDR-TB causes severe concerns from a public health perspective and constitutes a very demanding challenge for the national TB-control program. The drug resistance survey (DRS) carried out in the city of Minsk from November 2009 to December 2010 showed the highest ever reported incidence of MDR-TB. Almost 1 out of 2 (48%) of all infectious, smear-positive, pulmonary TB patients was shown to be infected with a MDR-TB strain. Both newly diagnosed and earlier treated patients had an outstanding high MDR incidence, 35.3% and 76.5%, respectively [2]. More recently, a nationwide survey was performed to investigate whether the high MDR incidence was limited to the capital or a more widespread problem. The results showed that the MDR-TB problem in Belarus is widely spread throughout the entire country [3]. It should be understood that this high prevalence of MDR-TB is not a problem limited to a certain city or country. Unpublished information from other parts of the former USSR indicates a broad problem with a high and increasing incidence of MDR-TB in various settings.

It is well documented that certain clones of resistant *Mycobacterium tuberculosis* strains are responsible for a significant proportion of the overall MDR-TB problem in Eastern Europe. Most often these clones belong to the so-called Beijing family of TB strains [4].

This study was the first attempt to increase the understanding of the *M. tuberculosis* strains responsible for the drug resistant TB in Belarus and its transmission. Pulmonary isolates from 81 MDR-TB patients in Minsk city were characterized by molecular strain typing, and the genotypes and level of clustering found were compared with a corresponding number of pan-susceptible clinical isolates.

As a first step, spoligotyping was used to identify clusters and the different geno-families of the studied strains. To further characterize the major spoligo-clusters seen in MDR-TB, the *pncA* gene – the gene where mutations related to resistance to the drug pyrazinamid (PZA) are found – was sequenced. In selected cases, the results were confirmed with MIRU/VNTR. The use of this sequencing for molecular epidemiology of PZA-resistant *M. tuberculosis* strains is an interesting option since the resistance-related point mutations are scattered all over the *pncA* gene with no specific mutations being commonly found [5].

Study population and methods

Consecutive isolates from culture-verified cases of pulmonary TB in Minsk were collected between 2009 and 2010 at the Belarusian National Reference Laboratory (NRL) in Minsk. Isolates found to be either susceptible to all tested first-line drugs (RIF, INH, streptomycin and ethambutol) or MDR (resistant to at least RIF and INH) were included in the study. The study totally comprised 163 clinical isolates from patients with pulmonary TB in Minsk city, 81 isolates being MDR and 82 pan-susceptible.

All strains were isolated at the Belarusian NRL in Minsk on Lowenstein–Jensen (LJ) medium and identified with standard biochemical tests. The *in vitro* susceptibility testing (DST) was carried out with the absolute concentration method on LJ medium with the following critical concentrations: INH 1 mg/L, RIF 40 mg/L, ethambutol 2 mg/L and streptomycin 4 mg/L. DST at the NRL in Minsk was externally quality assured by the WHO Supranational Reference Laboratory at the Swedish Institute for Communicable Disease Control (SMI) in Stockholm.

The molecular characterization of the isolates was carried out at SMI to identify clusters and the different geno-families of the *M. tuberculosis* strains.

All isolates were sub-cultured on LJ medium at SMI. For spoligotyping, mycobacterial lysates were prepared by re-suspending two 10 μl loops of bacteria in 250 μl of 1× TE buffer. After heat-killing the bacteria at 80 °C for one hour, the suspensions were centrifuged at 13,000 rpm for two minutes. The supernatants were discarded and the pellets re-suspended in 500 μl of 150 mM NaCl. This centrifugation and suspension steps were repeated. The final pellet was then dissolved in 25 μl of 1× TE buffer.

Thereafter, all isolates were genotyped with spoligotyping according to the standard protocol [6] using a commercial kit (Isogen Bioscience, BV Maarsen, The Netherlands). Briefly, the DR region of the TB genome was amplified using primers DRa and DRb, and the amplified biotinylated products hybridized to a set of 43 oligonucleotides covalently bound to a membrane. The hybridized PCR products were then incubated with streptavidin–peroxidase conjugate and the membrane exposed to chemo luminescence (Amersham ECL Direct™ Nucleic Acid Labeling and Detection System, GE Healthcare Limited, UK) and exposed to X-ray film (Amersham Hyperfilm™ ECL, GE Healthcare Limited, UK) according to the manufacturer's instruction. The X-ray film was developed using standard photochemical procedures after 20 min exposure. The DNA extracts of the *M. tuberculosis* reference strain H37Rv and of *Mycobacterium bovis* BCG were used as controls.

The patterns obtained were analyzed using Bionumerics software version 5.1 (Applied Maths, Belgium). A cluster was defined as two or more strains sharing identical spoligotyping patterns. Spoligotypes in binary format were converted to an octal code for comparison with the SITVIT2 proprietary database of the Pasteur Institute of Guadeloupe.

The octal codes found were entered in the SITVIT2 database, which is an updated version of the previously released SpolDB4 database [7]. In this database, SIT (Spoligotype International Type) designates spoligotypes shared by two or more patient isolates included in the database. Major phylogenetic clades were assigned according to signatures provided in the database, which defined 62 genetic lineages/sub-lineages. The SITVIT2 contains more than 3000 SITs with global genotyping information on about 74,000 *M. tuberculosis* isolates from over 160 countries of origin.

The *pncA* gene was sequenced in 76 MDR and 78 pan-susceptible isolates as earlier described [5]. Confirmatory MIRU/VNTR analysis was performed in selected strains in the Beijing and T1 clusters of MDR-TB isolates.

Results

Both the proportion of strains in a cluster (28% vs 16%), and the size of the largest clusters (42% vs 30%), were higher

among the isolates from MDR-TB patients. Among the MDR-TB isolates, two major clones, a specific T1 clone (SIT No. 266) and the Beijing clone (SIT No. 001), were most prevalent. These two were shown to cause 31% and 42% of all MDR-TB cases respectively. Among the pan-susceptible isolates, only small clusters of strains were seen with the exception of the Beijing type isolates (SIT No. 001) found in 30% of the cases. One more uncommon Beijing spoligotype (SIT No. 265) was also detected in susceptible isolates. A difference was observed also in the proportion of strains with unique patterns; among the MDR isolates, only 13 of 81 isolates (16%) had a unique pattern, compared with 23/82 (28%) for the pan-susceptible strains. The spoligotypes detected in the present study were compared with the SITVIT2 database, where most of the isolates could be identified as one of the lineages already recorded. For both the MDR and the susceptible group, most isolates were shown to belong to the Beijing family, seen in 33/81 (40.7%) and 26/82 (31.7%) respectively. Beside the Beijing geno-family, the T1 family was most commonly seen and identified in 30/81 (37.0%) and 16/82 (19.5%) of resistant and susceptible cases respectively. A statistical significant association to MDR-TB was demonstrated for the T1 type with the Fisher's exact test (p-value 0.0151), but not for the Beijing family (p-value 0.2564).

Discussion

The high proportion of strains in a cluster and the large cluster size seen in this study clearly indicate a pronounced ongoing transmission of drug resistant TB and especially of certain drug resistant clones of *M. tuberculosis*. The high transmission of MDR-TB strains was further supported by the fact that unique patterns were much more rarely seen in MDR isolates compared with isolates from patients with pan-susceptible TB.

It was remarkable that among all T1 isolates the most frequent spoligotype (SIT No. 266) was exclusively seen in MDR-TB. A clear majority of all T1 MDR isolates had this specific spoligopattern, 25/30 (83.3%), but it was not seen in a single case of pan-susceptible TB. This cluster in itself explains the significant difference observed for T1 isolates between MDR and susceptible *M. tuberculosis* isolates.

This study further investigated this finding, and since detailed information on clustering is not possible using spoligotyping as the sole method, it was decided to compare the T1 and Beijing family clones of the MDR-TB isolates by examination of the presence and distribution of *pncA* gene mutations. The results obtained clearly demonstrated a difference between these two major spoligo-clones. All 22 tested strains in the SIT No. 266 cluster had the same specific *pncA* mutation (Asp94Gly) and constituted a true and very closely related cluster. This is in contrast with the MDR Beijing spoligo-cluster SIT No. 001, where several different *pncA* patterns were seen among the tested isolates reflecting a much more heterogeneous group of isolates. The largest cluster detected in this group comprised eight Beijing strains with an identical *pncA* mutation (Trp68Gly) showing a sub-cluster of highly related strains. This difference between the SIT No. 266 and SIT No. 001 clusters was further confirmed in selected cases by MIRU/VNTR (data not shown).

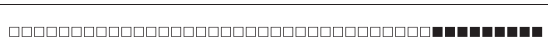
Finally, it can be noted that the spoligotype pattern obtained in 78/81 (96.3%) MDR isolates could be found in the international database, while non-reported patterns were much more commonly seen among the susceptible strains 15/82 (18.3%).

Patient's records were analyzed to investigate if a common source of infection or an epidemiological link could be identified among the 25 MDR-TB patients with the strongly homogeneous T1 SIT No. 266 clone. No such source or any common epidemiological connection could be identified. The patients comprised of 19 men and six women were born between 1946 and 1992, all 20 for whom HIV-status were known were HIV-negative. Three men had a history of imprisonment. The lack of a detectable link between these 25 individuals suggests that this clone is not restricted to a single epidemiological closely linked outbreak, but rather represents a widely transmitted resistant clone, frequently seen in and causing new cases of MDR-TB in Minsk city. This specific T1 strain was earlier reported only rarely in the Spol DB 4 database, isolated mainly in Russia (10 cases), but also in the USA (2 cases), as well as Latvia and Algeria with 1 isolate each. For the other SIT types identified, the actual numbers of observations were too low to draw any conclusions. Some, e.g. the H1 and T2 types, were seen only in susceptible isolates, while the two MANU type isolates were recognized only among the MDR isolates (Table 1). The most prevalent spoligotypes for the MDR and susceptible isolates respectively are shown in Fig. 1.

The high prevalence of the Beijing genotype found in this study, altogether 59/163 isolates (36.2%), as well as the connection between the Beijing genotype and MDR-TB, is well in line with earlier reports from the former USSR showing a high overall prevalence of Beijing TB: e.g., 34% in the Ukraine [8], 49% in Murmansk [9], 66% in Moscow [10], 50% in St. Petersburg [11], 46% in Kaliningrad [4], 42% in Estonia [12], and 54% in Latvia [13]. Regarding the T1-family, this is on the other hand a new finding, not earlier reported as such an important geno family, and especially the presence of a specific clone (SIT No. 266) restricted to the MDR cases was a totally new and unexpected finding.

Table 1 – Lineages of MDR and susceptible *M. tuberculosis* isolates from Minsk.

Lineage	MDR strains		Susceptible strains	
	No.	%	No.	%
Beijing	33	40.7	26	31.7
T1	30	37.0	16	19.5
SIT No. 266	25	30.9	0	0
LAM 9	6	7.4	4	4.9
H1	0	0	4	4.9
T2	0	0	5	6.0
Manu 2	2	2.5	0	0
H4	2	2.5	1	1.2
U	2	2.5	3	3.7
T5_RUS1	1	1.2	2	2.4
T4_CEU1	0	0	2	2.4
Others	2	2.5	4	4.9
Not earlier reported	3	3.7	15	18.3
Total	81	100	82	100

SIT	Spoligotype Description	Octal Number	Number (%), resistant strains	Number (%), susceptible strains	Clade*
1		000000000003771	33 (40.7)	25 (30.5)	Beijing
266		7757400037607 71	25 (30,9)	0 (0)	T1
53		777777777760771	1 (1.2)	12 (14.6)	T1
42		777777607760771	5 (6.1)	4 (4.8)	LAM9
47		777777774020771	0 (0)	4 (4.8)	H1
52		777777777760731	3 (3.7)	0 (0)	T2
254		7777600077607 71	1 (1.2)	2 (2.4)	T5_RUS1
39		7777773477604 71	0 (0)	2 (2.4)	T4_CEU1
118		777767777760771	0 (0)	2 (2.4)	T2
1482		7757400037637 71	2 (2.5)	0 (0)	Manu2

* Clade designations according to SITVIT2 using revised SpolDB4 rules

Fig. 1 – Description of the most prevalent spoligotypes in Minsk. Here showing the isolates belonging to the largest clusters among the samples that were matching a preexisting shared type in the SITVIT2 database.

The results of a molecular characterization of MDR-TB isolates compared with fully susceptible strains can reveal information useful for the national TB control program. The result of this study from Belarus clearly demonstrated differences in the population structure of strains isolated from patients with MDR- and pan-susceptible TB. More importantly, a very pronounced ongoing transmission of MDR-TB strains belonging to the T1 and Beijing families was also found to be responsible for a majority of all resistant cases in Minsk city. This finding highlights the need for a strengthened infection control to limit transmission of already resistant isolates of *M. tuberculosis*.

It is clear that most of the MDR problem today in Minsk, where almost every second patient with infectious TB is carrying a MDR-TB strain [2], is related to transmission and insufficient infection control rather than poor treatment/adherence causing many additional cases of acquired MDR-TB.

Apart from revealing important epidemiological information, the sequencing of the *pncA* gene also indicates the level of resistance to PZA, since these mutations in a very strong majority of cases are related to such resistance. Almost 80% of all studied MDR-TB isolates carried *pncA* mutations, which suggest an overall level of PZA resistance in the order of 75%. In contrast, over 90% of susceptible isolates showed wild type *pncA* sequences indicating PZA susceptibility. Some of the few mutations detected in PZA susceptible strains are silent or known not to confer PZA resistance.

In summary it can be concluded that this study demonstrated a high degree of clustering and the presence of large clusters among clinical isolates from MDR-TB patients. This indicates that the major cause of MDR-TB in Minsk is an ongoing transmission of already drug-resistant *M. tuberculosis* strains belonging to a limited number of specific clones rather than the independent development of MDR-TB in a substantial number of patients, initially infected with pan-susceptible strains. As expected, the Beijing strains were prevalent, but also the T1 family of *M. tuberculosis* strains were shown to be responsible for a large portion of all MDR-TB cases in Minsk city. Using sequencing of the *pncA* gene, the Beijing cluster could be further divided into several sub-clusters, while a specific T1 clone (SIT No. 266) was confirmed to be one strictly homogenous cluster responsible for almost one third (25/81) of the MDR cases examined.

Early detection of cases with resistant TB is of crucial importance to make possible timely modification of treatment to effective drug combinations and directed infection control measures to limit further transmission of MDR-TB.

Conflict of interest

None declared.

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